

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 500

**Supplemental Assay Method for the Determination of Phenol in Veterinary
Biologics (pullorum antigen, *Mycoplasma synoviae* antigen, and *Mycoplasma
gallisepticum* antigen)**

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Supplemental Assay Method for the Determination of Phenol in Veterinary Biologics (pullorum antigen, *Mycoplasma synoviae*, and *Mycoplasma gallisepticum* antigen)

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1. Introduction

The Code of Federal Regulations, Title 9 (9 CFR) (Animals and Animal Products) states that the Animal and Plant Health Inspection Service (APHIS) is responsible for administering the Virus-Serum-Toxin Act. It specifies methods for testing phenol in tuberculin (9 CFR 113.406), pullorum tube antigen (9 CFR 113.407), and *Mycoplasma gallisepticum* antigen (9 CFR 113.408) products. This document describes the phenol testing procedures for pullorum tube antigen, *Mycoplasma synoviae* and *Mycoplasma gallisepticum* antigen. The procedure for phenol in tuberculins is described elsewhere in **SAM 513** and **SAM 514**.

2. Materials

2.1 Equipment/instrumentation

- 2.1.1** Balance, analytical, capable of measuring 0.0001 g
- 2.1.2** Balance, top loading, capable of measuring 0.01 g
- 2.1.3** Volumetric pipettes, Class A, meet ASTM Standard E969-83
- 2.1.4** Volumetric flasks, Class A, with barrel head glass stopper, meet ASTM E288 requirements
- 2.1.5** Erlenmeyer flasks, 125-ml
- 2.1.6** Buret with PTFE stopcock, 10-ml, precision bore, calibrated to ASTM E-694 accuracy requirements
- 2.1.7** Buret with PTFE stopcock, 50-ml, precision bore, calibrated to ASTM E-694 requirements
- 2.1.8** Graduated cylinders [meets ASTM D86, D216, and D447 requirements], 50-, 100-, 250-, 500-, and 1,000-ml
- 2.1.9** Glass-stoppered Erlenmeyer flasks, 250-ml
- 2.1.10** Heating/stirring plate with stirring bars
- 2.1.11** Fast filter paper, Whatman No. 1
- 2.1.12** Disposable beaker, 5-ml

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2.1.13 Rubber stopper, No. 1

2.1.14 Small spot light lamp

2.2 Reagents/supplies

All chemicals are reagent grade. Use distilled or demineralized water or water of equivalent purity.

2.2.1 Methyl orange--Purity: 98.0%

2.2.2 Silicotungstic acid ($\text{H}_4[\text{Si}(\text{W}_3\text{O}_{10})_4] \cdot 26\text{H}_2\text{O}$)--Purity: 99.0% Store at 4°C.

2.2.3 Arsenic trioxide (As_2O_3)--Purity: 99.9%

2.2.4 Sodium bicarbonate (NaHCO_3)--Purity: 99.9%

2.2.5 Potassium bromate (KBrO_3)--Purity: 98.5%

2.2.6 Potassium bromide (KBr)--Purity: 99.0%

2.2.7 Phenol ($\text{C}_6\text{H}_5\text{OH}$)--Purity: $\geq 99.0\%$

3. Preparation for the test

3.1 Personnel qualifications/training

No special test-related training is needed for this testing. Analysts performing this procedure should first conduct 2 trial runs using controls and standards and obtain results within acceptable limits.

3.2 Preparation of equipment/instrumentation

No special equipment is utilized other than routine setup and cleaning.

3.3 Preparation of reagents (all reagents stable for at least 6 months unless specified)

3.3.1 20% HCl: Slowly add 200 ml HCl to 600 ml H_2O ; dilute to 1 L. Store at room temperature.

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3.3.2 0.1% methyl orange: Add 0.1 g methyl orange to 100 ml H₂O. Filter if necessary. Store at room temperature.

3.3.3 Silicotungstic acid solution (SAS): Dissolve 60 g H₄[Si(W₃O₁₀)₄]*26H₂O in 400 ml H₂O in 500-ml volumetric flask. Add 50 ml H₂SO₄. When cool, dilute to volume with H₂O. Store at room temperature.

3.3.4 Clarifying solution (CS): Add 50 ml SAS and 125 ml 20% HCl to 325 ml H₂O. Prepare fresh prior to each test.

3.3.5 "Acid solution" for As₂O₃ standard solution: Add 110 ml HCl and 2.5 ml methyl orange solution to 100 ml H₂O. Store at room temperature.

3.3.6 0.0500 N As₂O₃: Dissolve 2.4730 g dried As₂O₃ in 25 ml hot 1N NaOH in 1-L volumetric flask. Neutralize it with 25 ml 1N H₂SO₄. Cool and dilute to volume with H₂O. Store at room temperature.

Caution: As₂O₃ is extremely toxic, avoid contact; handle in fume hood using gloves, mask, and goggles. Consult Material Safety Data Sheet for specific handling instructions.

3.3.7 Phenol standard, 0.25%: Dissolve 2.50 g phenol in 1.00 L H₂O. Store at room temperature.

Critical Control Point: The final diluted volume of the test fluid must be adjusted as described in Section 3.3.2.8.

3.3.8 Test fluid (TF): Dissolve 0.30 g NaHCO₃, 1.67 g KBrO₃, and 15.00 g KBr in H₂O and Q.S. to 1 L with H₂O. Store at room temperature. The TF volume must be adjusted by adding corrected volume of H₂O to TF. It must take a volume of 21.3 ml to titrate 25 ml 0.050 N As₂O₃ in 10 ml "Acid Solution." A first time titration will require less than 21.3 ml TF. Adjust as described in the following example:

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Example: Assume the first time titration volume is 20.5 ml

$$(1,000 \text{ ml of TF}) - (20.5 \text{ ml}) = 979.5 \text{ ml}$$

$$\frac{(979.5) (\text{desired vol})}{(\text{actual vol})} \text{ or } \frac{(979.5) (21.3)}{(20.5)} = 1,017.7 \text{ ml}$$

For corrected volume of H₂O:

$$1017.7 - 979.5 = 37.2 \text{ ml to be added to TF.}$$

Note: TF in buret has to be put back into flask.

3.4 Preparation of the sample

3.4.1 Receipt

Follow sample receipt procedures as described by standard Section operating procedures.

3.4.2 Preparation

Licensed or prelicense biologics products are generally received in sealed serum bottles and stored at 4°C prior to testing. Before testing, allow sample vials and reagents to warm to room temperature.

4. Performance of the test

4.1 Pullorum tube antigen

(Analyze the control pool and phenol standard each time testing is performed. Analyze control and standard in duplicate, and samples in triplicate.)

4.1.1 Add 5 ml sample and 50 ml 20% HCl to 250-ml glass-stoppered flask. Shake until the solution decolorizes (final appearance will be white-cloudy, typically takes 2-3 minutes). Add 50 ml H₂O and mix. Filter 50 ml through filter paper.

4.1.2 Transfer 50 ml to another flask. Add 1 drop methyl orange, stopper and shake a few seconds. Observe the color; when red, go to **Section 4.1.3**.

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4.1.3 Titrate with 2 ml test fluid (TF), stopper and shake a few seconds. Observe the color. When red, repeat **Section 4.1.3**. When colorless, go to **Section 4.1.4**.

4.1.4 Shake 30 seconds. Add 1 drop indicator, stopper and shake a few seconds. Observe the color. When it does not turn to colorless within 10 seconds, titrate with 1 ml TF, stopper and repeat **Section 4.1.4**. When colorless, go to **Section 4.1.5**.

4.1.5 Shake 1 minute. Add 1 drop indicator, stopper and shake a few seconds. Observe the color. When red stays longer than 10 seconds, titrate with 0.50 ml TF, stopper and repeat **Section 4.1.5**. When colorless, record total volume of TF as the endpoint of titration and use for calculation of percent phenol.

4.2 *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antigen

(Analyze the control pool and phenol standard each time testing is performed. Analyze control and standard in duplicate or triplicate, and samples in triplicate.)

4.2.1 Add 5 ml sample and 100 ml CS to 250-ml glass-stoppered flask. Shake 2 minutes. Filter through filter paper.

4.2.2 Transfer 50 ml filtrate to another flask. Add 1 drop methyl orange, stopper and shake a few seconds. Observe the color; when red, go to **Section 4.2.3**.

4.2.3 Titrate with 2 ml test fluid (TF), stopper and shake a few seconds. Observe the color. When red, repeat **Section 4.2.3**. When colorless, go to **Section 4.2.4**.

4.2.4 Shake 30 seconds. Add 1 drop indicator, stopper and shake a few seconds. Observe the color. When it does not turn to colorless within 10 seconds, titrate with 1 ml TF, stopper and repeat **Section 4.2.4**. When colorless, go to **Section 4.2.5**.

4.2.5 Shake 1 minute. Add 1 drop indicator, stopper and shake a few seconds. Observe the color. When red stays longer than 10 seconds, titrate with 0.50 ml TF, stopper and repeat **Section 4.2.5**. When colorless, record total volume of TF as the endpoint of titration and use for calculation of percent phenol

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5. Interpretation of the test results

5.1 Pullorum tube antigen (Report average of triplicates)

% Phenol = (volume of test fluid) x (0.04)

Satisfactory Phenol Content: $0.55 \pm 0.05\%$.

5.2 *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antigen (Report average of triplicates)

% Phenol = (volume of test fluid) x (0.04)

Satisfactory Phenol Content: $0.25 \pm 0.05\%$.

5.3 Controls

Results for controls and standards must be within acceptable limits; otherwise repeat testing.

6. Report of test results

Report results of the test(s) as described by Section standard operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.407-408, U.S. Government Printing Office, Washington, DC, 2005.